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Does agricultural use of azole fungicides contribute to resistance in the human pathogen *Aspergillus fumigatus*?

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Abstract

Azole resistance in human fungal pathogens has increased over the past twenty years, especially in immunocompromised patients. Similarities between medical and agricultural azoles, and extensive azole (14α-demethylase inhibitor, DMI) use in crop protection, prompted speculation that resistance in patients with aspergillosis originated in the environment. *Aspergillus* species, and especially *Aspergillus fumigatus*, are the largest cause of patient deaths from fungi. Azole levels in soils following crop spraying, and differences in sensitivity between medical and agricultural azoles (DMIs), indicate weaker selection in cropping systems than in patients receiving azole therapy. Most fungi have just one CYP51 paralogue (isozyme CYP51B), but in *Aspergillus* sp. mutations conferring azole resistance are largely confined to a second paralogue, CYP51A. Binding within the active centre is similar for medical and agricultural azoles but differences elsewhere between the two paralogues may ensure selection depends on the DMI used on crops. Two imidazoles, imazalil and prochloraz, have been widely used since the early 1970s, yet unlike triazoles they have not been linked to resistance in patients. Evidence that DMIs are the origin, or increase the frequency, of azole resistance in human fungal pathogens is lacking. Limiting DMI use would have serious impacts on disease control in many crops, and remove key tools in anti-resistance strategies.
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Keywords: aspergillosis; *Aspergillus fumigatus*; azoles; CYP51; DMI fungicides; drug resistance; itraconazole; paralogue; sterol 14α-demethylase

1 INTRODUCTION

The last forty years have witnessed the development and commercialisation of a diverse group of sterol biosynthesis inhibitors, which have revolutionised control of fungal diseases in crops, animals and humans. Early products were derivatives of piperidine, pyridine, pyrimidine and morpholine chemistry, but nowadays the market is dominated by at least sixty imidazoles and triazoles (azoles), and which account for around 25% of fungicide use worldwide. Although not all are widely used today, azoles inhibit the pivotal 14α-demethylase step in the sterol biosynthesis pathway, which ensures that they offer good control of many diseases caused by true fungi, but not oomycetes.

In a clinical setting, azoles are used to control a number of pathogens, yeasts and dermatophytes. Chief amongst these are facultative yeasts belonging to *Cryptococcus*, and especially *Candida*, which cause uncomfortable infections of mucous membranes, and especially the vagina, and which may be fatal for immunocompromised patients. Ubiquitous spores of *Aspergillus* species, and especially *Aspergillus fumigatus* (Fresen.), are inhaled, but are effectively dealt with by the immune system in heathy individuals. But for patients with ongoing lung problems, infection within pre-existing lung cavities results in a condition known as chronic pulmonary aspergillosis,[1] and which requires long-term therapy to contain the disease. For immunocompromised and HIV–AIDS patients, the fungus may become invasive, spreading from lung cavities to other organs. Untreated aspergillosis, and especially invasive aspergillosis, is usually fatal and is the largest cause of deaths in patients due to fungi.

Azoles are single-site inhibitors and so are not immune from the evolution of resistance, especially in pathogens that sporulate profusely and have short generation times. Indeed, resistance evolved in the 'high risk' cereal powdery mildews within four years after introduction of triadimefon and triadimenol in the late 1970s.[2] Resistance also emerged rapidly in *Candida* species,[3] but it is resistance in the pathogen *A. fumigatus* that currently causes much concern. The medical azole itraconazole was introduced during the 1980s, and the first resistant isolate was cultured from an American patient with invasive aspergillosis in 1989.[4] Other reports of resistance followed, and in 1998 in The Netherlands, isolates were recovered from a number of patients which combined a point mutation with a tandem promoter repeat (TR_{34}L98H), and which conferred cross-resistance to the available azoles.[5] Resistance is now worldwide, and the mortality rate of patients with resistant invasive aspergillosis is twice (88%) that of patients with azole-sensitive strains.[6] It is well established that resistance can evolve in patients requiring long-term azole treatment.[5,7] Because resistant isolates were recovered from some patients requiring treatment for aspergillosis, but with no prior history of azole therapy, it was suggested that selection for resistance can occur in the environment where *A. fumigatus* occurs

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Table 1. Degradation of three triazoles in two soils following a single spray applied to spring barley[a]

	Sandy loam			Clay loam		
Triazole	Initial MEC (mg kg^{-1} soil)	Half-life (days)	Undetected (days)	Initial MEC (mg kg^{-1} soil)	Half-life (days)	Undetected (days)
Propiconazole	0.3	20	400	0.4	30	700
Epoxiconazole	0.35	20	800	0.4	130	800+
Triadimenol	0.35	50	700	0.4	200	700

[a] Data summarised from Bromilow *et al.*[16] MEC, maximum exposure concentration.

in soils and decaying organic wastes.[8,9] This focused attention on use of azoles in agriculture and horticulture, and the possibility that they have driven selection and spread of resistance.[6,10] Similar concern had been expressed earlier by Serfling *et al.*,[11] following evidence of cross-resistance between medical triazoles and the 14α-demethylase inhibitor (DMI) tebuconazole in laboratory-generated resistant isolates of the maize pathogen *Colletotrichum graminicola*, which also causes cutaneous mycosis in humans. This has echoes of concern for antibiotic resistance, and use of antibiotics in livestock production. Already some azoles are threatened in the EU with withdrawal on health grounds, because they may be endocrine inhibitors, and adding a 'resistance' issue may strengthen moves to ban key fungicides.

This short perspective article adds to a number of earlier papers on the role of environmental selection.[12–15] It examines, from the viewpoint of crop protection, that use of azole fungicides is a contributory factor in the increased detection of resistance in *A. fumigatus*, and considers the impact that loss of azole fungicides might have on disease control.

2 HOW DO AZOLE FUNGICIDES ENTER THE ENVIRONMENT?

Azoles are used in wood and textile preservation and consumer and animal health products, but their major use is in agriculture, horticulture and prevention of post-harvest losses. The imidazole imazalil was introduced in the late 1970s, and is still widely used today in wax formulations applied as post-harvest treatments to citrus and pome fruits, bananas and seed potatoes. Another imidazole, prochloraz, was introduced a few years later, primarily for control of cereal eyespot (*Tapesia* sp.) and is still used today. Several triazoles were introduced in the 1980s, especially for control of cereal and fruit diseases, and although they are still in use in some markets, they have largely been replaced by more effective products, including propiconazole (1990), tebuconazole (1992), difenconazole (1994) epoxiconazole (1994) and bromuconazole (2000). It was the introduction of these five triazoles that Dutch workers correlated with the first detection of azole-resistant TR_{34}L98H *A. fumigatus* isolates.[9]

3 BEHAVIOUR OF AZOLES IN SOILS FOLLOWING SPRAYING OF FIELD CROPS

Degradation of three triazoles in two soil types over two years was charted by Bromilow *et al.*,[16] and their results are summarised in Table 1. Between 30 and 38% of a single spray (0.5 kg ha^{-1}) applied each year to a young spring barley crop resulted in maximum exposure concentrations (MECs) of between 0.3 and 0.4 mg kg^{-1} in the top 10 cm of each soil. Triazoles degrade slowly and the half-life of each triazole varied between 20 and 200 days depending on soil type, and it was after more than two years before soils were azole free. These MEC values are somewhat higher than those calculated by Gisi,[13] but are very much lower than MEC values (11 mg L^{-1} blood serum[4]) in patients receiving daily oral treatment for aspergillosis. Equating 1 kg with 1 L, these results, together with sensitivity data (Table 2), suggest that selection for resistance is unlikely to occur in soils at least following spray treatments. Azoles are moderately lipophilic, are strongly adsorbed to soil organic matter and are quickly unavailable to soil microflora, or for selection for resistance. Although *Aspergillus* spp. are commonly found, especially in subtropical and warm temperate soils,[17] there are limited data on the occurrence of *A. fumigatus*. It is a saprophyte growing on decaying organic matter, but is seldom recovered from straw which is a common surface residue in cropping systems.[18]

Table 2. Sensitivity of medical and agricultural azoles to *Aspergillus fumigatus*[a]

Azole	Itraconazole-sensitive range EC 50 (µg mL^{-1})	Itraconazole-resistant range EC50 (µg mL^{-1})	Ref.
Medical triazoles			
Itraconazole	0.125–1.0	4.0–32.0	9
	0.13–0.23	2.0–16.0	20
Voriconazole	0.50–4.0	2.0–32.0	9
	0.13–0.5	0.5–16.0	20
Posaconazole	0.031–0.25	0.25–1.0	9
	0.030–0.5	0.06–2.0	20
DMIs			
Imazalil	0.125–0.5	1.0–8.0	9
	0.03–0.25	0.06–8.0	20
Prochloraz	0.125–0.5	1.0–32.0	9
	0.06–0.5	0.13–16.0	20
Propiconazole	2.0–8.0	16.0–32.0	9
	2.0–8.0	16.0–≥16.0	20
Difenoconazole	1.0–4.0	8.0–32.0	9
Epoxiconazole	2.0–16.0	32.0	9
Bromuconazole	1.0–4.0	16.0–32.0	9
Tebuconazole	1.0–8.0	8.0–16.0	9

[a] The results bring together sensitivity data from two large European surveys involving isolates from both clinical and environmental sources. The same microdilution broth assay method was used in both surveys.[17]

4 SENSITIVITY OF *A. FUMIGATUS* TO AZOLE FUNGICIDES

In vitro studies have examined the sensitivity of *A. fumigatus* isolates from the environment, including cropping areas, to azoles

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using the standard microdilution broth method CCLS (M38-A).[19] One extensive Dutch study[9] identified imazalil as the most active azole, and which also showed cross-resistance with itraconazole. Five triazoles introduced in the 1990s were all less active in controlling *A. fumigatus* (Table 2), and some (e.g. cyproconazole, myclobutanil) are reported essentially inactive.[13] An earlier survey of 150 Swiss isolates from environmental sources, also including cropping areas, presented a somewhat different picture.[20] Both imazalil and prochloraz were the most active agricultural azoles and showed cross-resistance with itraconazole (resistance factor 200+), whereas propiconazole, although cross-resistant (resistance factor 8), was very much less active than the imidazoles. A more recent laboratory study involving both clinical and environmental isolates of *A. fumigatus* confirmed that prochloraz selected for resistance,[21] even at a concentration below the MEC in soils shown in Table 1. Taken together, these results show that imidazoles, which were being used in crop protection well before the emergence of TR_{34}L98H resistance, could have selected for azole resistance.

Table 3. Genotype changes in azole-resistant *A. fumigatus* isolates from different origins

Resistance mechanism	Genotype alteration	Origin	Ref.[a]
Target-site modification	Amino acid change G54E/L/K/V/R, L98H, Y121F, P216L, I138L/R, M220Y/K/T/V/W. T289A, Y431C, Y434C, Y443G, G448S	Clinic	5,7,13,26,39
	G54E/K/R/W, G138C, P216L	Laboratory	9,22,30
	L98H, Yi2iF, T289A	Environment	8,10,25
Increased *cyp51A* expression	Promoter insert TR34, TR46[b]	Clinic	9,26,49,50
	TR34, TR46	Environment	8,10,42
Increased efflux	Gene involved *atrF, cdr1B*	Clinic	24,25
	Afmdr3, Afmdr4	Laboratory	22

[a] References include reviews which should be consulted for sources of the original data.
[b] TR, tandem repeat.

5 WHAT CAN MUTATIONS AND OTHER GENOTYPE CHANGES TELL US ABOUT RESISTANCE?

Laboratory selection for resistance in *A. fumigatus* generally results in increased efflux of azoles[22] and similar multidrug resistance occurs in isolates from patients receiving long-term azole therapy.[23–25] Target site mutations (single nucleotide polymorphisms, SNPs) generally confer much higher levels of resistance than multidrug resistance. Promoter changes that increase production of the target CYP51 protein enhance resistance further if combined with point mutations, and both changes are essential for cross-resistance between all medical triazoles.[5] The most common SNP coupled with a tandem repeat in the promoter is TR_{34}L98H (Table 3), which has been cultured in many countries from both clinical and environmental sources.[8,10,26] At least 18 other SNPs, some coupled with a different tandem repeat in the promoter, have been found in clinical, but not in environmental, isolates.[27] Only two amino acid changes, Y121F and Y431C, are homologous with the very large number of SNPs linked to azole resistance in other agricultural and human fungal pathogens.[28]

Understanding a possible reason for this difference between *A. fumigatus* and other fungi is complicated by the presence in fungi of up to three paralogues (isozymes) of the *cyp51* gene, *cyp51A*, *cyp51B* and *cyp51C*, which is *Fusarium* species specific and does not code for a functional sterol 14α-demethylase.[29] *A. fumigatus* has two *cyp51* paralogues that have 63% amino acid sequence identity.[30] *cyp51A* is involved in azole resistance[30,31] and *cyp51B* is primarily responsible for sterol 14α-demethylation[29] and has a role in fungal growth and development.[3] Although resistance has been linked to overexpression of *cyp51B*,[24] in *A. fumigatus* tandem repeats and SNP mutations linked to resistance occur only in its *cyp51A* paralogue. In some fungi one *cyp51* paralogue may be functionally inactive, although its activity is quickly restored if ergosterol levels are depleted.[32,33] Targeted disruption of *A. fumigatus cyp51* paralogues suggests that recovery from the impact of azoles on the *cyp51B* paralogue by activation of *cyp51A* is possible,[30] as was shown in *Fusarium graminearum*.[29] Increased expression of CYP51A paralogues, from otherwise low levels, in response to DMI fungicides was also observed in the barley pathogens *Rhynchosporium commune*[33] and *Pyrenophora teres* f.sp. *teres*.[34] Although a CYP51 protein was generated when each *A. fumigatus* paralogue was transformed into a yeast expression vector,[35] the paralogue constructs used in these experiments were cDNAs and expression was driven by a yeast *cyp51*, and not an *A. fumigatus*, promoter. So when under control of its own promoter, *A. fumigatus cyp51A* may be redundant until activated by reduced ergosterol levels.

Different *cyp51* paralogues certainly can have a role in the evolution of azole resistance.[32,33] Azoles bind more tightly to CYP51B than to CYP51A,[31] and this difference between the two enzymes provides a possible explanation for why resistance mutations in *A. fumigatus* are paralogue specific. Despite evidence of low-level overexpression of *cyp51B* in some resistant isolates,[24] azole targeting of CYP51B may reduce ergosterol levels to a point when normal metabolic controls call upon *cyp51A* to restore normal ergosterol levels. But this can only happen if effective azole resistance mutations are selected in *cyp51A*. Different DMI fungicides are known to preferentially select particular resistance mutations,[36,37] but clearly corresponding mutations in *cyp51B* which confer significant resistance are either not selected by agricultural triazoles in *cyp51A*, or carry a fitness penalty when expressed in CYP51A.

6 EVOLUTION OF RESISTANCE

The genetic relatedness among azole-resistant *A. fumigatus* isolates has been explored by microsatellite fingerprinting using two, three and four tandem repeat markers.[38] Isolates included in surveys were generally cultured from hospitalised patients, some of whom had received therapy for aspergillosis over several years. The overall conclusions from these surveys were that, despite being closely related in many cases, each isolate had a unique genotype indicating that they were not transmitted from patient to patient, and had evolved independently from different originally sensitive isolates.[7,9,31] The largest survey involved 144 TR_{34}L98H itraconazole-resistant isolates selected from 3847 isolates cultured from patients in Dutch hospitals between 1994 and 2009.[9] Plotting the number of new resistant genotypes as a function of time, the authors calculated a rate of change of 1.37 ± 0.05 genotype mutations each year, which pointed to emergence of the first TR_{34}L98H isolate between 1994 and 2000. In fact TR_{34}L98H was first identified in 1998. It seems no isolates cultured before

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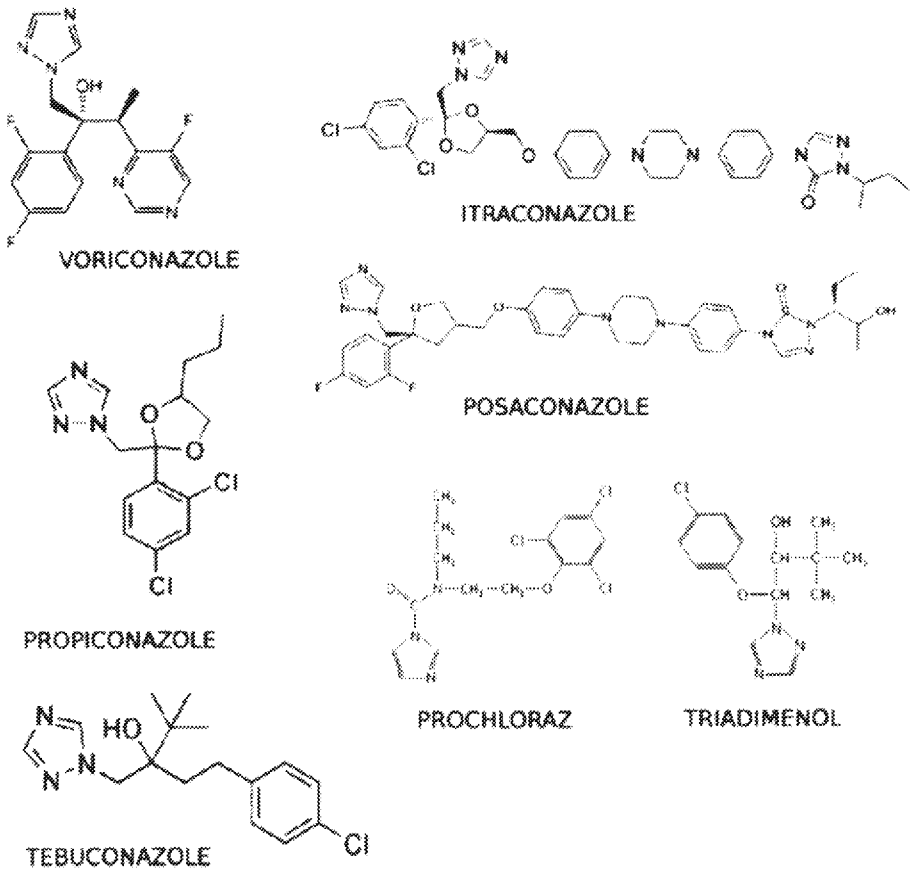

Figure 1. Medical (itraconazole, voriconazole, posaconazole) and agricultural (propiconazole, tebuconazole, prochloraz, triadimenol) azoles.

1994 were available, so if this azole resistance mechanism was present before then, this conclusion may be different. However, the authors link the emergence of TR_{34}L98H in the 1990s with selection by five DMIs authorised for use on Dutch farms during the 1990s. The analysis assumes that all isolates originated in The Netherlands, but fingerprinting covering large parts of the genome is not suitable for identifying the geographic origin of isolates, unless isolates from geographic origins outside The Netherlands were included.[39]

The origin of genotype changes conferring resistance to at least one medical triazole, and associated with different resistance mechanisms, is shown in Table 3. Other target-site mutations in *cyp51A* have been identified, but are not linked with resistance as they occur in both resistant and sensitive isolates.[24] Laboratory studies involving selection for resistance are limited, but several surveys have analysed genotype changes in isolates from both the clinic and the environment. These surveys suggest that evolutionary changes involving different resistance targets are far greater in the clinic than in the agricultural environment, which probably reflects higher selection pressure in patients receiving azole therapy than occurs in field crops. Nevertheless, this suggests that the vast majority, if not all, of clinical isolates are not the results of fungicide use in crop protection.

7 MOLECULAR INTERACTION OF AZOLES WITH CYP51A

Medical and agricultural azoles (Fig. 1) have similar three-dimensional structures but very different activities against *A. fumigatus*, and computational modelling has been used to explain these differences.[9,30] Although a crystal structure of *A. fumigatus* CYP51B has recently been published,[40] no crystal structure of *A. fumigatus* CYP51A exists, so human and *Mycobacterium tuberculosis* CYP51A crystal structures, which have a 38 and 24% amino acid sequence identity respectively with *A. fumigatus* CYP51A, were used to derive a homology model of the *Aspergillus* enzyme. Azoles occupy space within the heme binding pocket forming van der Waals contact and hydrogen bonds with tyrosine and serine residues lining the pocket, and coordinating with heme iron, preventing oxidation of the lanosterol 14α-methyl group. Both itraconazole and voriconazole occupy the expected 'pose' within the binding site, confirming the validity of the homology model, at least in the region of the heme binding pocket.[9] Twenty DMI fungicides considered to share a core structure with medical triazoles were computationally 'docked' within the CYP51A homology model. Propiconazole and bromuconazole showed the most similar 'pose' with itraconazole and tebuconazole and epoxiconazole were the most similar to voriconazole. A fifth DMI, difenconazole, was also considered sufficiently similar as it blocked the hydrophobic access channel, a position occupied by the long tail of itraconazole and voriconazole. A similar modelling study involving *Zymoseptoria tritici* (= *Mycosphaerella graminicola*) confirmed these binding properties of triazole agricultural DMIs.[41]

Because of their similar binding properties, and their significant cross-resistance with medical azoles, Snelders *et al.* concluded that selection by these five DMIs contributed to the origin of *A. fumigatus* resistant isolates in the environment.[9] This echoes

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conclusions derived from fingerprinting analysis discussed above. The robustness of these conclusions needs some qualifications. Interaction with leucine 98, which is not in the active site of the enzyme, was not even discussed. Although factors such as permeability can affect activity, generally the most active and resistant compounds offer the strongest selection for resistance. The most active agricultural DMIs against *A. fumigatus* are imidazoles (Table 2), yet prochloraz was not included in the 'docking' studies, and imazalil was dismissed as it retained activity against TR_{34}L98H isolates. Bioassay data in their paper present a different picture. A range of MIC_{50} values show that there were no differences in resistance factors (= 16) between the two imidazoles and the five triazole DMIs. Attempts to generate TR_{34}L98H resistance by *in vitro* selection were unsuccessful, but a similar resistance combining a promoter insert and point mutations (TR_{45}Y1212F/T298A) has since been generated in the laboratory by selection with increasing concentrations of several agricultural fungicides.[42]

8 IS THERE A PROBLEM?

The occurrence of azole resistance in *Aspergillus* spp. that cause aspergillosis is generally based on isolates cultured from patients already in hospital, so it is not clear what proportion of patients entering hospital are already infected with azole-resistant strains, and what proportion become infected with resistant strains whilst in hospital. An early extensive survey confirmed that resistance developed during prolonged azole therapy.[43] In a later study involving 14 patients with itraconazole resistance (not TR_{34}L98H), in all but one patient clinical observation indicated that resistance evolved in their lungs after hospitalisation,[7] and was not due to infection from an agricultural source. Azole-resistant isolates are certainly present in the environment, but so far there has been little focus on their occurrence in association with crop and animal production systems. A recent Chinese survey of soil samples from 144 greenhouses recovered 73 *A. fumigatus* isolates, of which two were resistant to voriconazole and one to itraconazole.[42] The authors give no information on cross-resistance with DMIs, nor do they state if these three isolates were from soils beneath crops treated with agricultural DMIs, which would not necessarily be the fungicides used to control many of the diseases of the 18 different crops involved. Molecular diagnostics capable of detecting mutations can be applied to DNA from soil or the airspora, in hospital and field environments. Resistance mutations occurring at frequencies as low as 1×10^{-8} can now be detected easily.[44] Such research has yet to be carried out, but must be before scientifically based changes in the use of azoles in crop protection can be considered.

Some cross-resistance between medical and agricultural azole resistance is not surprising because they have the same mode of action and resistance mechanisms. The risk of resistance emerging depends on many factors,[45] including how azoles are used in practice. The cornerstone of anti-resistance strategies involves use of products with effective, but different, modes of action. In clinical practice, only echinocandins offer a realistic, but perhaps less effective, alternative mode of action for aspergillosis treatment. Nevertheless, they should be considered, either as a mixture partner, or in alternation with azoles, in strategies aimed at delaying further evolution of resistance in *A. fumigatus*. For crop disease control several different modes of action are available to partner azoles. Consequently, for foliar (but not seed) treatments agricultural DMIs are generally used as prepacked mixtures, with chlorothalonil, QoIs, SDHIs or MBCs. Apart from MBCs,[9] there is no information on the activity of these other fungicides on *Aspergillus* spp. causing aspergillosis. Such information is needed before the environment can be considered as a source of clinically resistant isolates of *A. fumigatus*. Confronted with these different modes of action in the agricultural environment may well reduce the risk of azole resistance in *Aspergillus* spp. as well as in the target plant pathogens.

9 CONCLUSIONS

Several years have now elapsed since it was suggested that use of agricultural (DMI) fungicides might be linked to increased resistance to medical azoles,[8] but so far no critical evidence has emerged to support this claim. Mutation is a fundamental property of living organisms, and azole-resistant mutations will inevitably arise in environmental populations of *Aspergillus* spp., although their frequency will depend on the relative fitness of resistance mechanisms. Spread of resistance also depends on the genetic systems within environmental populations of *A. fumigatus*, but is likely to be very limited. *Aspergillus fumigatus* (syn. *Neosartorya fumigata*, O'Gorman, Fuller and Dyer) has a sexual cycle,[46] but little variation is generated by recombination.[39,47] Fungi also lack plasmids, so transfer within populations via plasmids, carrying resistance alleles, as happens with antibiotic resistance, cannot occur. Critically, no evidence has yet been published from appropriate field experiments that not only detect resistant isolates within field crops, but also show that their frequency increases, especially in the airspora, following foliar sprays. Loss of agricultural DMIs would not only have serious consequences for crop yields, but would also remove a valuable partner in anti-resistance strategies and contribute to the unnecessary loss of key modes of action. Until evidence is presented confirming that selection for resistance by agricultural DMIs in the environment contributes to increasing difficulties in controlling aspergillosis diseases in the clinic, and it is not a reflection of the increasing number of immunocompromised patients receiving azole therapy, there seems no need to alter the way agricultural DMIs are used in crop protection. Despite their single site mode of action, anti-resistance strategies, combined with introduction of new triazoles over the years, have maintained the effective contribution of azoles to crop protection for over forty years.

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